Plant Pathology (2011) **60**, 719–729 Doi: 10.1111/j.1365-3059.2011.02428.x

Genetic structure and diversity of *Phakopsora pachyrhizi* isolates from soyabean

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Simple sequence repeat (SSR) markers were used to classify 116 isolates of *Phakopsora pachyrhizi*, the cause of soyabean rust, collected from infected soyabean leaves in four agroecological zones in Nigeria. A high degree of genetic variation was observed within the sampled populations of *P. pachyrhizi*. Eighty-four distinct genotypes were identified among three of the four agroecological zones. Nei's average genetic diversity across geographical regions was 0·22. Hierarchical analysis of molecular variance showed low genetic differentiation among all populations of *P. pachyrhizi*. The majority (> 90%) of the genetic diversity was distributed within each soyabean field, while approximately 6% of the genetic diversity was distributed among fields within geographic regions. Low population differentiation was indicated by the low F_{ST} values among populations, suggesting a wide dispersal of identical genotypes on a regional scale. Phylogenetic analysis indicated a strictly clonal structure of the populations and five main groups were observed, with group II accounting for 30% of the entire population. Because of the asexual reproduction of *P. pachyrhizi*, single-step mutations in SSR genotypes are likely to account for the genetic differences within each group.

Keywords: *Glycine max*, population biology, soyabean, soyabean rust, SSR genotype, virulence phenotype

Introduction

Soyabean rust, caused by *Phakopsora pachyrhizi*, impacts global soyabean (*Glycine max*) production in part because of its wide distribution in the major soyabean-producing countries and the rapid reduction in green leaf area and premature defoliation that correlate negatively with yield and yield components (Hartman *et al.*, 1991). In Nigeria, soyabean rust was first reported in 1999 (Akinsanmi *et al.*, 2001) and it is now endemic in most soyabean-producing areas in the country. Considerable research has been done in Nigeria to identify sources of resistance to soyabean rust (Twizeyimana *et al.*, 2007, 2008) and to document the major pathotypes of *P. pachyrhizi* present in soyabean production areas (Twizeyimana *et al.*, 2009).

Breeding for resistance has been hampered because *P. pachyrhizi* can rapidly overcome the major resistance genes *Rpp1–4* (Hartman *et al.*, 2005). There are numerous examples of the ineffectiveness of these four single dominant genes when challenged with isolates of *P. pachyrhizi* from different continents (Bonde *et al.*, 2006; Paul & Hartman, 2009; Pham *et al.*, 2009). Along with single-gene resistance, partial resistance to soyabean rust has also been described (Hartman *et al.*, 2005). Partial resistance may be expressed as reduced uredinia number and size, a longer latent period, and/or other components related to reduced fungal reproduction. This type of resistance may be more durable or effective against all *P. pachyrhizi* isolates than single resistance genes. Knowledge of the dominant pathotypes in a region and whether they predominate or change over time may allow for the deployment of cultivars with specific resistance to the prevalent *P. pachyrhizi* pathotypes. The other approach is to promote partial resistance based on selection criteria for quantitative resistance that may not be pathotype-specific (Ribeiro *et al.*, 2007). Nonetheless, because durability of host-plant resistance is an evolutionary process, breeding and deployment of durable resistance would be more effective in the management of soyabean rust if the genetic structure of the *P. pachyrhizi* population were known. Such information would, for example, allow early-generation breeding lines to be screened against a range of pathogen genotypes reflecting the genetic diversity of the *P. pachyrhizi* population in a given area.

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Published online 17 February 2011

Plant Pathology

Fungal plant pathogens possess a range of mechanisms through which genetic variation may be generated, including sexual recombination, migration, spontaneous mutation and somatic hybridization (McDonald *et al.*, 1989). In this regard, *P. pachyrhizi* is only known to reproduce by asexual production of urediniospores on soyabean and other hosts (Ono *et al.*, 1992), which presumably would limit its genetic variation. The amount and distribution of genetic variation within and among populations is an important factor in understanding the population biology of pathogenic fungi, but little is known for *P. pachyrhizi*. Genetic structure can be used to deduce the impact of different forces that influence the evolution of a pathogen population. In turn, this provides a better understanding of evolutionary forces that may allow prediction of the potential for pathogen populations to evolve in agricultural ecosystems (McDonald, 1997). For instance, a large amount of genetic diversity distributed over a small spatial scale suggests the possibility of rapid adaptation by a pathogen to a changing environment, such as new host resistance genes or fungicides. Conversely, a high degree of genetic similarity among populations collected from widely separated geographic regions suggests the occurrence of significant long-distance dispersal and gene flow.

Gene flow over long distances poses a threat to deployment of resistance genes tailored to local pathogen populations, because immigrants may possess virulence genes that can overcome resistance in local host cultivars. In addition, new virulence genes also might be incorporated into local pathogen populations through recombination (Brasier, 1988). Little is known about the genetic variation in populations of *P. pachyrhizi* from soyabean, mainly because appropriate molecular markers were previously lacking. Recently, highly polymorphic microsatellite markers from *P. pachyrhizi* genome sequence data were developed (Anderson *et al.*, 2008), enabling a study of the population biology of this fungus. While previous studies identified three to 18 pathotypes of *P. pachyrhizi* worldwide (Bromfield *et al.*, 1980; Yamaoka *et al.*, 2002), the degree of genetic variation in *P. pachyrhizi* populations is unknown.

Seven pathotypes of *P. pachyrhizi* infecting soyabean have been reported in the Derived Savanna (DS), Northern Guinea Savanna (NGS) and Southern Guinea Savanna (SGS) agroecological zones in Nigeria (Twizeyimana *et al.*, 2009), with the highest level of pathogenic variation in the DS zone. The extent of genetic variation among these pathogenic groups was unknown, weakening predictions on the geographic durability of the putative resistant soyabean genotypes. Thus, the objectives of this study were to (i) characterize the genetic variation in *P. pachyrhizi* populations collected from different sites in different agroecological zones in Nigeria, and (ii) compare the virulence profile and SSR genotypes of *P. pachyrhizi* populations found in Nigeria.

Materials and methods

Isolate collection and purification

A total of 116 isolates collected from soyabean in ten states of Nigeria located in the DS, Humid Forest (HF), NGS and SGS agroecological zones (Table 1, Fig. 1) were used in this study. All isolates were collected between 16 October and 8 November 2005. A field collection of isolates consisted of one to several leaves bearing uredinia from a single plant in a field.

Approximately 20 mg of urediniospores were harvested from all leaves from each location or sampling site using a cyclone spore collector (G-R Manufacturing Company) and placed in 1·8-mL cryogenic vials. The vials were stored in liquid nitrogen and transported to the International Institute of Tropical Agriculture (IITA) plant pathology laboratory in Ibadan. To purify the *P. pachyrhizi* collection, urediniospores were used to inoculate healthy detached leaves (Twizeyimana *et al.*, 2007) of a highly susceptible soyabean cultivar TGx 1485-1D grown in the greenhouse. Prior to inoculation, frozen urediniospores were heat shocked at 40°C for 5 min, and hydrated by incubating over water in an enclosed Petri dish overnight (Frederick *et al.*, 2002). When uredinia started to erupt (about 9–13 days after inoculation), urediniospores from a single isolated uredinium (i.e. without any uredinia close by) were picked using a sharp needle under a stereoscopic binocular at ×40, and mixed with 0·3 mL sterile water. Using a fine brush, the resulting spore suspension was then used to inoculate newly detached leaves. Single-urediniospore isolates were derived after three cycles of single uredinial transfers to establish purified cultures. Adequate quantities of single-urediniospore cultures of each collection were obtained by inoculating several detached leaves of susceptible soyabean cv. TGx 1485-1D (Twizeyimana *et al.*, 2008) with respective culture collections. Urediniospores from each isolate were used to inoculate a set of host differentials to study the virulence variation (Twizeyimana *et al.*, 2009) and characterize the molecular genotypes among 116 *P. pachyrhizi* isolates.

Virulence diversity

Urediniospores from each isolate were used to inoculate detached leaves (Twizeyimana *et al.*, 2007) of eight

Table 1 Summary of the sources of *Phakopsora pachyrhizi* isolates used to establish the genetic diversity in the rust populations from soyabean in Nigeria

Agroecological zone	Number of locations	Number of isolates
Derived Savanna	44	52
Humid forest	1	3
Southern Guinea Savanna	16	26
Northern Guinea Savanna	24	35
Total	85	116

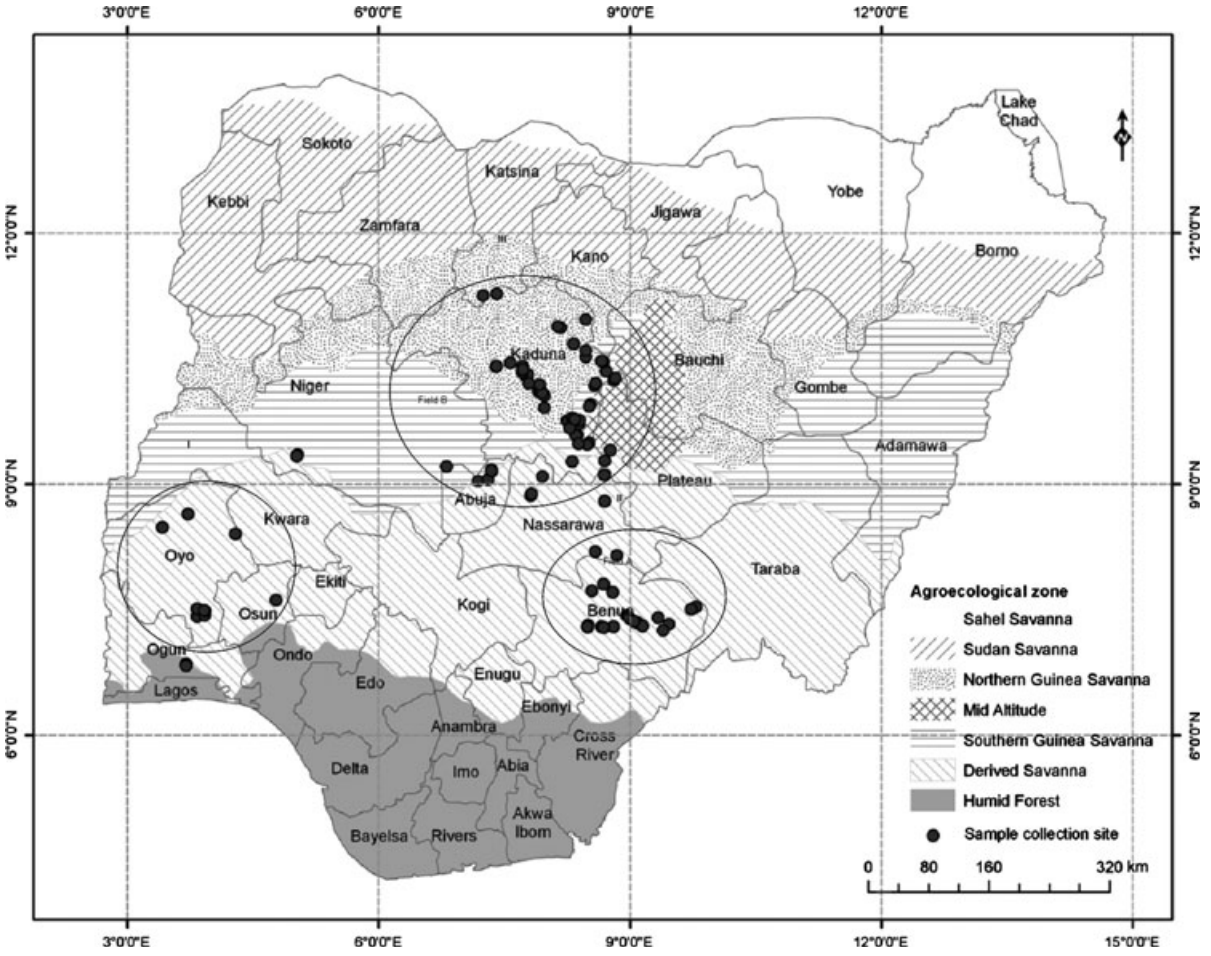

Figure 1 Geographical locations of soyabean fields in agroecological zones in Nigeria where *Phakopsora pachyrhizi* was sampled from soyabean in 2005. Names in the map are names of states of Nigeria. Circles represent major soyabean growing areas.

soyabean genotypes selected as a set of host differentials. The set comprised four soyabean accessions with the *Rpp* resistance genes [PI 200492 (*Rpp1*), PI 230970 (*Rpp2*), PI 462312 (*Rpp3*), and PI 459025B (*Rpp4*)], two highly resistant genotypes containing unknown resistance genes (PI 594538A and UG-5) and two highly susceptible genotypes (TGx 1485-1D and TGx 1844-4F) (Twizeyimana *et al.*, 2009). The total number of uredinia per cm^2 of leaf tissue was recorded for subsequent analyses. Each isolate treatment was replicated three times in a single run, and two independent runs were conducted.

Molecular genotypes

Genomic DNA was isolated from approximately 20–30 mg of urediniospores of each isolate using the Qiagen DNeasy Plant Mini kit. Quantitative PCR (qPCR) with *P. pachyrhizi* -specific primers Ppm1 and Ppa2 and FAM probe (Frederick *et al.*, 2002) was performed on extracted DNA to confirm the identification of *P. pachyrhizi* and for the DNA quantification. For the qPCR procedure, a Stratagene MX3005P^{TM} thermocycler with ROX reference dye was used. The final reaction volume of 25 μL included 10 μL eluate, and was assayed using Platinum^{TM} qPCR Supermix UDG (Invitrogen) with each primer at 300 nM, probe at 100 nM and $MgCl_2$ at 7 mM. All samples were assayed in duplicate. The cycling regimen was an initial 2 min at 60°C incubation, a subsequent 2 min at 95°C denaturation, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Fluorescence was recorded at the end of each cycle. Cycle threshold values were determined by the Strategene X3005P^{TM} thermocycler software using an amplification-based threshold, and quantification of the DNA was estimated by comparison to the reference dilution series obtained by following these steps: a DNA reference standard was prepared from a 0·05% Tween 20 suspension of concentrated, freshly heat-killed spores quantified using a haemocytometer, then extracted using the FastDNA^{TM} kit, adjusted to 51·2 spore equivalents (SEq) μL^{-1}, and then serially diluted 1:8 with 1 μg mL^{-1} salmon sperm DNA in 5 mM Tris, pH 8, to a low-end concentration of 0·0125 SEq μL^{-1}. One SEq is the amount of genomic DNA from a single

dikaryotic spore of *P. pachyrhizi* (Haudenshield & Hartman, 2011).

Primer sequences and the PCR conditions used to amplify the 18 SSR markers were described previously (Anderson *et al.*, 2008). Briefly, for SSR amplification, a 12·5-μL reaction mixture containing 1× Phusion HF buffer (New England Biolabs), 2·0 mM $MgCl_2$, dNTPs at 200 nM each, 0·2 U Phusion polymerase (New England Biolabs) and 100 SEq DNA was assayed in a PTC-100 thermocycler (MJ Research), with initial denaturation at 95°C for 30 s, followed by 30 or 35 cycles of 94°C for 30 s, 65°C for 30 s and 72°C for 60 s, plus a final extension at 72°C for 10 min (Anderson *et al.*, 2008). The forward primers were labelled with HEX (hexachlorofluorescein) and the resulting amplification products were separated and detected by capillary electrophoresis on an ABI3130x1 Analyzer (Applied Biosystems, Inc.), using ROX500 as the internal size standard. Peak calls were made with GENEMARKER v1.51 software and individual isolates were scored visually for each SSR locus.

Data analysis

The total number of uredinia per cm^2 of leaf tissue data from the virulence diversity study of 116 isolates were pooled over runs for final analysis as no heterogeneity was detected when both runs were subjected to ANOVA to test for homogeneity of error variance. The distribution of total number of uredinia was established using the PROC UNIVARIATE procedure of SAS (version 9.1, version 9.1.3; SAS Institute). Three uredinia classes were defined based on the departure from the grand mean for number of uredinia across all isolates and genotypes: the first (HV: highly virulent), the second (V: virulent) and the third (LA: less virulent) consisted of uredinia numbers that were > 1 standard deviation above the grand mean, plus/minus one standard deviation around the grand mean, and < 1 standard deviation below the grand mean, respectively (Twizeyimana *et al.*, 2009).

Microsatellite alleles detected as length polymorphisms and scored using the GENEMARKER v1.51 software were compiled into binary data for all loci based on the presence (=1) or absence (=0) of alleles. The percentage of polymorphic loci, the observed number of alleles, the effective number of alleles and Nei's genetic diversity (Nei, 1973) were calculated using population genetic analysis software (POPGENE, version 1.31; Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Canada). The SSR genotypes by region were calculated using ARLEQUIN version 2.0 (University of Geneva, Switzerland). Analysis of molecular variance (AMOVA) was performed using GENALEX6 (Peakall & Smouse, 2006) to estimate the variance components among and within *P. pachyrhizi* isolates across different spatial scales: agroecological zones (DS, NGS and SGS) and states (Benue, Federal Capital Territory, Kaduna, Niger, Nasarawa, Oyo and Plateau). A pairwise population matrix of Nei's genetic distance (Nei, 1972) based on allele frequencies by region and population pairwise values of population differentiation (F_{ST}) and their *P*-values were calculated using GENALEX6. Isolates from HF zones were not included in these analyses because of the small sample size.

Phylogenetic analyses were performed using the Phylogenetic Analysis Using Parsimony package (PAUP*, version 4.0b10; Sinauer Associates, Inc.). Heuristic searches for the most parsimonious trees were conducted with random stepwise addition (1000 replications) and branch-swapping algorithm using tree bisection-reconnection. Support for each branch in the inferred trees was evaluated by 1000 bootstrap replications. Three additional isolates were included in these analyses; these were two isolates, US-FL and US-IL, collected respectively from soyabean in Florida and Illinois, USA in 2007 and one isolate, TW72-1, obtained from soyabean in Taiwan in 1972.

The length of the most parsimonious tree reconstructed from the data using PAUP* was compared to the distribution of tree length obtained in 10 000 randomized datasets (Burt *et al.*, 1996; Hovmøller *et al.*, 2002; Enjalbert *et al.*, 2005). The lengths of trees constructed from the randomized datasets were estimates of the lengths of trees of individuals sampled from a freely recombining population. In this method, the null hypothesis, i.e. the population sampled has undergone recombination, would be rejected if the observed tree length values were not within the distribution of randomized values.

A genetic distance matrix derived from SSR allele differences of all pairs of isolates and a matching matrix derived from differences in virulence phenotype between isolates were generated using GENALEX6. These matrices were correlated using the Mantel correlation coefficient of GENALEX6.

Results

Virulence diversity

Seven pathotype groups were found in 116 Nigerian *P. pachyrhizi* isolates based on the virulence data recorded on four genotypes (PI 200492, PI 459025B, TGx 1485-1D and TGx 1844-4F). Pathotypes 1 and 2 accounted for 84% of all isolates. Pathotype 3 isolates were the most virulent, while isolates in pathotype 4 were the least virulent (Table 2).

Single sequence repeat genotypes

In all, 47 alleles were amplified by 18 SSR primer pairs from the *P. pachyrhizi* isolates collected from Nigeria. There were a total of 84 SSR genotypes among all isolates collected from the three agroecological zones, with DS and SGS zones having the highest and lowest number of 34 and 20 genotypes, respectively (Table 3). The NGS zone had an intermediate number of genotypes with 30 SSR genotypes.

Table 2 Virulence levels of seven pathotypes found among *Phakopsora pachyrhizi* isolates collected in different agroecological zones (AEZ) in Nigeria

Pathotype[a]	Number of isolates	Origin of isolates[b]	Virulence on differential genotypes[c]							
			TGx1485-1D	TGx1844-4F	PI 200492 (*Rpp1*)	PI 459025B (*Rpp4*)	PI 230970 (*Rpp2*)	PI 462312 (*Rpp3*)	UG-5	PI 594538A
1	70	DS-B (15), DS-F (1), DS-KD (7), DS-NS (5), DS-OS (1), DS-OY (8), HF-OG (1), NGS-KD (25), SGS-F (2), SGS-PL (5)	HV	V	LV	LV	LV	LV	LV	LV
2	28	DS-B (4), DS-NS (1), HF-OG (2), NGS-KD (17), SGS-NG (1), SGS-OY (2), SGS-PL (1)	V	V	LV	LV	LV	LV	LV	LV
3	7	DS-B (3), DS-OS (1), SGS-F (1), SGS-NG (2)	HV	HV	LV	LV	LV	LV	LV	LV
4	3	DS-NG (2), SGS-KW (1)	V	LV	LV	LV	LV	LV	LV	LV
5	4	DS-KD (1), NGS-KD (1), SGS-F (1), SGS-KD (1)	HV	V	V	LV	LV	LV	LV	LV
6	2	DS-B (2)	HV	V	V	V	LV	LV	LV	LV
7	2	DS-NS (1), NGS-KD (1)	HV	V	LV	V	LV	LV	LV	LV

[a]The three virulence levels on differential genotypes were used to assign isolates into pathotypes.
[b]Isolate origin with three parts: (i) name of AEZ (DS: Derived Savanna, HF: Humid Forest, NGS: Northern Guinea Savanna and SGS: Southern Guinea Savanna), (ii) state of origin (B: Benue, F: Federal Capital Territory, KD: Kaduna, KW: Kwara, NG: Niger, NS: Nasarawa, OG: Ogun, OS: Osun, OY: Oyo and PL: Plateau), and (iii) total number of isolates in brackets.
[c]Level of virulence correspond to three uredinia classes obtained following the distribution of total number of uredinia obtained by the PROC UNIVARIATE analysis in SAS. The three virulence levels were: HV (highly virulent), V (virulent) and LV (less virulent) and consisted of uredinia numbers that were > 1 standard deviation above the grand mean, plus/minus one standard deviation around the grand mean, and < 1 standard deviation below the grand mean, respectively.

Table 3 Genotypic variation and Nei's genetic diversity statistics from 18 simple sequence repeat (SSR) loci of *Phakopsora pachyrhizi* isolates collected from three agroecological zones (AEZ) in Nigeria[a]

AEZ	No. of isolates	SSR genotypes	Polymorphic loci (%)	Observed no. of alleles	Effective no. of alleles	Nei's genetic diversity
Derived Savanna	52	34	78·7	1·79 (0·41)[b]	1·26 (0·26)	0·18 (0·15)
Southern Guinea Savanna	26	20	85·1	1·85 (0·36)	1·31 (0·31)	0·20 (0·16)
Northern Guinea Savanna	35	30	68·1	1·68 (0·47)	1·38 (0·33)	0·23 (0·18)
Combined AEZ	113	84	87·2	1·87 (0·34)	1·33 (0·28)	0·22 (0·15)

[a]Isolates from HF zones were not included in these analyses because of the small sample size.
[b]Values in parentheses are standard errors.

Table 4 Analysis of molecular variance among and within *Phakopsora pachyrhizi* isolates from three agroecological zones (AEZ) in Nigeria

Source	d.f.	Sum of squares	Mean squares	Observed partition		F_{ST}[a]	*P*-value[b]
				Variance	Total (%)		
Among AEZ[c]	2	28·998	14·499	0·267	5·4	0·052	0·002
Among states in AEZ	3	25·282	8·427	0·318	6·0	0·081	0·022
Within fields	104	487·420	4·687	4·742	90·4	0·053	0·003

[a]Fixation index, a measure of population differentiation.
[b]Probability of obtaining more extreme random variance component and F_{ST} than the observed values by chance alone.
[c]Hierarchical analysis based on the 113 isolates of *P. pachyrhizi* collected from Derived, Northern Guinea and Southern Guinea Savanna agroecological zones.

The percentage of polymorphic loci recorded in the *P. pachyrhizi* populations from three agroecological zones was 87%. The *P. pachyrhizi* population collected from the SGS zone had the highest percentage of polymorphic loci (85%), while the population from the NGS zone had the lowest percentage (68%). The average number of observed alleles per locus in the three agroecological zones was 1·87, with SGS having a higher number of alleles per locus than either the DS or NGS zones. However, the effective number of alleles was higher for isolates collected in the NGS zone than for those collected from the SGS or DS zones (Table 3).

Population genetic variation and pairwise comparison

Nei's genetic diversity for all agroecological zones was 0·22, with NGS and DS having the highest and lowest values, respectively (Table 3). Results from AMOVA revealed significant ($P \leq 0{\cdot}01$) genetic differentiation and a high genetic variation of *P. pachyrhizi* populations within agroecological zones (Table 4). The variance associated with this genetic variation within field populations in agroecological zones was 90·4%. The corresponding variance associated with genetic variation of *P. pachyrhizi* populations among the agroecological zones was low (5·4%). Little genetic differentiation was found among *P. pachyrhizi* populations in agroecological zones and states, as shown by the very low F_{ST} values (range 0·052–0·081) (Table 4).

Pairwise comparisons of F_{ST} values across agroecological zones were generally low and with varying degrees of statistical significance. The lowest F_{ST} value was observed between the NGS and DS rust population pair (0·044), while the highest value was observed between the NGS and SGS population pair, (F_{ST} = 0·076) (Table 5). Unlike pairwise comparisons across agroecological zones, pairwise comparisons among states varied from low to moderate (Table 6). The lowest and highest genetic differentiation were observed in pairings between the Oyo and Benue populations (F_{ST} = 0·04) and the Nasarawa and Niger populations (F_{ST} = 0·33), respectively.

Table 5 Pairwise comparisons of Nei's genetic distance (above diagonal) and genetic differentiation (F_{ST})[a] (below diagonal) in *Phakopsora pachyrhizi* isolates from three agroecological zones in Nigeria

	Derived Savanna (DS)	North Guinea Savanna (NGS)	Southern Guinea Savanna (SGS)
DS	–	0·018	0·018
NGS	0·044**	–	0·034
SGS	0·045**	0·076***	–

[a]Genetic differentiation (F_{ST}) was calculated to compare populations pairs. Double (**) and triple (***) asterisks indicate F_{ST} values significant at $P < 0{\cdot}01$ and 0·001, respectively.

Phylogenetic analysis

Parsimony analyses performed on all SSR genotypes placed 106 Nigerian *P. pachyrhizi* isolates into five different groups. The assignment of the ten remaining isolates to any of the five groups was unclear (Fig. 2). Group II accounted for more isolates (30% of the entire

Table 6 Pairwise comparisons of Nei's genetic distance (above diagonal) and genetic differentiation (F_{ST})[a] (below diagonal) in *Phakopsora pachyrhizi* from seven states in Nigeria

State[b]	Benue	FCT	Kaduna	Niger	Nasarawa	Oyo	Plateau
Benue	–	0·068	0·021	0·079	0·052	0·020	0·050
FCT	0·129 ns	–	0·086	0·143	0·118	0·070	0·063
Kaduna	0·046*	0·163**	–	0·075	0·030	0·029	0·039
Niger	0·158*	0·228*	0·136*	–	0·129	0·087	0·087
Nasarawa	0·135*	0·315**	0·046 ns	0·333**	–	0·055	0·073
Oyo	0·039 ns	0·231*	0·070*	0·282**	0·262**	–	0·054
Plateau	0·088 ns	0·055 ns	0·049 ns	0·121 ns	0·196*	0·176*	–

FCT: Federal Capital Territory.

[a]Genetic differentiation (F_{ST}) was calculated to compare population pairs. Single (*) and double (**) asterisks indicate F_{ST} values significant at $P < 0·05$ and 0·01, respectively; ns, not significant.

[b]Isolates from three states (Ogun, Osun and Kwara) were not included in these analyses because of small sample size.

population) and had well-supported nodes compared to the other groups. This group was composed of isolates from all four agroecological zones, while the remaining groups consisted of isolates from the DS, NGS and SGS zones, except group III, which had isolates from only the NGS and SGS zones. All pathotypes, except for pathotype 7, were found in group II. However, only pathotypes 1 and 2 were found in group I (Fig. 2). The well-supported clade of two isolates from the USA (bootstrap support value of 98%) were in group I, as was isolate TW72-1 from Taiwan.

The tree estimated from the observed SSR dataset had a length of 197 steps, which was significantly ($P \leq 0·001$) shorter than the trees generated from a randomized dataset (mean of 738 steps, standard deviation = 14·3). This led to the rejection of the null hypothesis of free recombination and confirmed the significance of clonal reproduction of the Nigerian *P. pachyrhizi* population.

SSR genotype, virulence phenotype and geographical distance correlations

The phylogenetic tree did not show a clear relationship between SSR genotypes and geographical zones, an observation that also was supported by the lack of significance in the Mantel test of correlation ($P = 0·177$ with 10 000 permutations) between geographical and genetic distance matrices. The Mantel correlation coefficient using a genetic distance matrix derived from SSR allele differences of all pairs of isolates and a matching matrix derived from virulence phenotypes were significantly correlated ($r = 0·20$, $P = 0·004$). However, the phylogenetic tree did not indicate a clear relationship between SSR genotypes and virulence phenotypes, except for group I, which was comprised of isolates belonging to pathotype 1 and two isolates of pathotype 2 (Fig. 2).

Discussion

This study was undertaken to characterize the genetic variation in *P. pachyrhizi* populations collected from different agroecological zones in Nigeria. The results showed that populations of *P. pachyrhizi* in Nigeria are highly variable and that the vast majority of genetic variation was distributed within fields at a fine spatial scale. This is apparently the first report on genetic diversity and population structure in *P. pachyrhizi* infecting soyabean.

Using a hierarchical analysis of genetic diversity, the total genetic variation was partitioned among spatially separated populations of *P. pachyrhizi* from three agroecological zones in Nigeria; the geographical distances between these populations ranged from 0·5 to 800 km. Total genetic variation was partitioned into two components with the majority of genetic diversity (> 90%) distributed within each soyabean field, while approximately 6% of genetic diversity was distributed among fields. Urediniospores of *P. pachyrhizi* are wind-dispersed, and circumstantial evidence of migration over hundreds or thousands of kilometres has been reported (Isard *et al.*, 2005). This dispersal mechanism may be responsible for the limited genetic variation among field populations observed in this study. Other fungi with comparable levels of genetic differentiation and which disperse their genes over large geographic distances include *Stagonospora nodorum* (Keller *et al.*, 1997) and *Mycosphaerella graminicola* (Zhan *et al.*, 2003), with genetic differentiation values of 0·08 and 0·04, respectively. Most of the soyabean production in Nigeria is concentrated in the savannas, where several wild and edible legumes are present, such as kudzu (*Pueraria lobata*) and jicama (*Pachyrhizus erosus*). Both are potential over-seasoning hosts for *P. pachyrhizi* since they thrive even in the dry season (Tian *et al.*, 2000). Although the sources of inoculum have not been documented in West Africa, it is plausible that these wild and edible legumes may form a continuous host population throughout the savannas of Nigeria. If so, *P. pachyrhizi* urediniospores could move readily between local geographic populations of alternative hosts, maintaining a uniform source population for urediniospore inoculum that could infect soyabean fields each growing season, and this could explain the low genetic differentiation observed among fields at the state or agroecological scale.

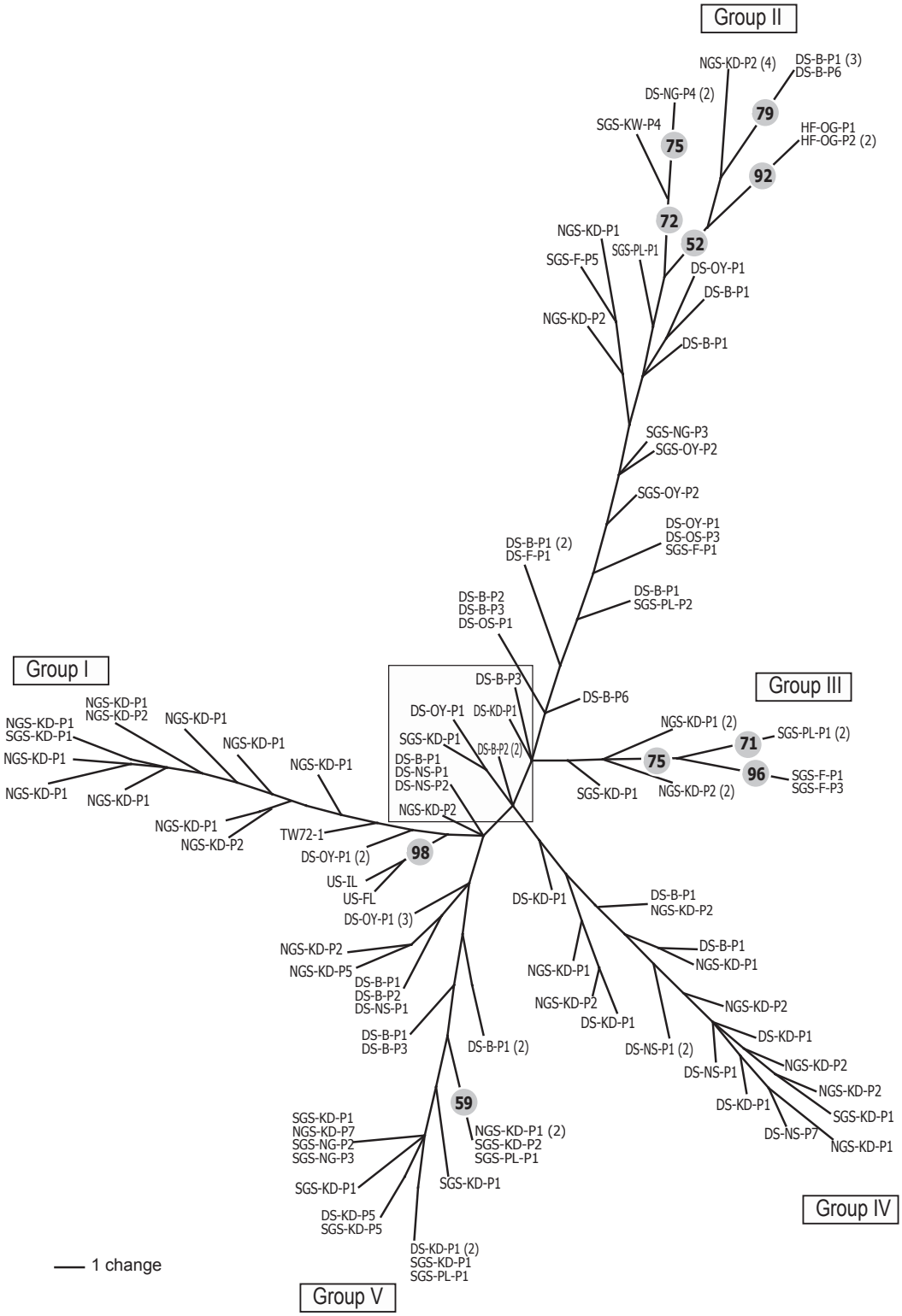

Figure 2 Consensus unrooted tree of 116 Nigerian, two USA and one Taiwanese (TW72-1) *Phakopsora pachyrhizi* isolates inferred from maximum parsimony analysis (PAUP*, version 4.0b10) with 80% majority rule. Numbers in dark circles on branches are bootstrap values > 50% in 1000 replicates. Isolate names are composed by three parts: (i) agroecological zone (AEZ) (DS: Derived Savanna, HF: Humid Forest, NGS: Northern Guinea Savanna and SGS: Southern Guinea Savanna), (ii) state of origin (B: Benue, F: Federal Capital Territory, KD: Kaduna, KW: Kwara, NG: Niger, NS: Nasarawa, OG: Ogun, OS: Osun, OY: Oyo and PL: Plateau), and (iii) pathotype number 1–7. Numbers in parentheses are total numbers of isolates from different locations. The ten isolates inside the box were not clearly assigned to any of the five groups.

The analysis of SSR diversity among 116 isolates is consistent with the strictly clonal nature of the Nigerian *P. pachyrhizi* population which was previously reported in general for this fungus based on the lack of a known sexual cycle (Ono *et al.*, 1992). Following the recent establishment of this fungus in Nigeria (Akinsanmi *et al.*, 2001), it was expected that *P. pachyrhizi* populations would have low genetic diversity among a limited number of clonal lineages as a result of recurrent founder events in which a small number of genotypes are selected by a strong selective agent, such as a new resistance gene or a new fungicide (Brown, 1994, 1995). However, the results of the present study showed moderate to high levels of genetic diversity in populations of *P. pachyrhizi* across agroecological zones as several distinct *P. pachyrhizi* genotypes were recovered from field populations over relatively small geographic areas. For example, in the NGS field population, 30 genotypes could be differentiated among 35 isolates analysed and 20 genotypes were differentiated among 26 isolates in the SGS population.

The high genetic diversity observed in the field populations of the soyabean rust pathogen that lacks a sexual recombination cycle may in part be explained by the introduction of exotic and genetically distinct isolates, followed by a mutational event, a phenomenon that has been reported for wheat rust pathogens (Keiper *et al.*, 2006; Wingen *et al.*, 2007; Hovmøller *et al.*, 2008). Like other rust pathogens where host–pathogen co-evolution is strongly dependent on cultivar dynamics, population evolution in *P. pachyrhizi* may occur by random mutations causing a shift from avirulence to virulence or *vice versa*. Such changes are believed to occur via a single-step mutation followed by host selection (Brown, 1994; Park *et al.*, 1995; Wellings *et al.*, 2000; Brown & Hovmøller, 2002; Hovmøller *et al.*, 2002; Justesen *et al.*, 2002). Furthermore, the high proportion of total genetic variation distributed within fields indicates that *P. pachyrhizi* populations have the potential to evolve relatively quickly to changing environments (McDonald *et al.*, 1989). Thus, the short useful lifetime of *Rpp* resistance genes (Bonde *et al.*, 2006; Paul & Hartman, 2009; Pham *et al.*, 2009) may be related to the ability of the fungus to evolve rapidly.

A consensus-unrooted most parsimonious tree shows the existence of five main groups in the Nigerian soyabean rust population. Members of the five groups were widely dispersed and, in some cases, resulted in the existence of several SSR genotype groups in the same field or in close proximity. This observation is supported by the low values of pairwise comparisons of Nei's genetic distance and genetic differentiation between states or zones. However, the greater divergence observed between some states than between zones may be caused by the local proliferation of particular clonal lineages. The Nigerian *P. pachyrhizi* population data were consistent with the long-distance divergence scenario in population genetics (Brown & Hovmøller, 2002) as it appears that the population may have been established by one clonal lineage that diverged after becoming established in Nigeria. The one isolate from Taiwan (obtained in 1972) and both isolates from the USA (obtained after the Nigerian collection of isolates) were part of group I. Since these three isolates from outside of Nigeria were clustered in one group, it seems likely that the other four clonal lineages may have been derived from Asia, as represented by the Taiwan isolate, and then diverged at some point between when it was first reported in Nigeria in 1999 (Akinsanmi *et al.*, 2001) and when the samples of the current study were collected in 2005. It is also possible that multiple introductions produced five distinct lineages, with one of these introductions being clearly associated with the Taiwan isolate. Another possibility is that at least some of these lineages resulted from mutations associated with the molecular markers that occurred in the Nigerian population following its establishment. In either case, these hypotheses cannot be rigorously tested using the present dataset, but with the additional analysis of other isolates from Asia it may have been possible to differentiate single or multiple introductions of *P. pachyrhizi* in Nigeria.

In a previous study on virulence diversity, seven distinct pathotypes of *P. pachyrhizi* were established, with isolates belonging to pathotypes 3 and 4 being the most and least virulent, respectively (Twizeyimana *et al.*, 2009). In the present study using the same 116 isolates, all pathotypes, except for pathotype 7, were present in group II, while only pathotypes 1 and 2 were found in group I. There was a relatively low correlation between virulence phenotypes and SSR genotypes. Lack of concordance between pathogenic and molecular variation data has been reported previously for other rust pathosystems (Brake *et al.*, 2001; Keiper *et al.*, 2006; Hovmøller & Justesen, 2007). Nonetheless, some specific inferences can be drawn from the present data. For example, isolates in pathotypes 1 and 2 were part of all five groups which would indicate that convergent pathotype evolution could occur from genetically distinct ancestors. Similar observations were reported for wheat yellow rust (Hovmøller *et al.*, 2002, 2008), the rice blast fungus (Levy *et al.*, 1993) and the field pea blight fungus, *Mycosphaerella pinodes* (Zhang & Fernando, 2003).

Analysis of the genetic structure of *P. pachyrhizi* showed that genetically diverse populations of the pathogen are responsible for the soyabean rust epidemics in Nigeria. This genetic variation is present within isolate populations irrespective of the geographic zone. These findings have direct implications on the management of soyabean rust. Deployment of resistant cultivars is the most sustainable and one of the most effective approaches to controlling soyabean rust. Because of the high levels of genetic diversity within populations, the expression of resistance to *P. pachyrhizi* may be dependent upon the pathogen population. As such, it is recommended that several isolates or a broad selection of rust isolates be used to screen germplasm effectively for resistance in a given geographic region. The results of this initial study on the genetic diversity and population structure of *P. pachyrhizi* on soyabean provide valuable

information for future work on the population biology of *P. pachyrhizi* and can serve as a baseline for understanding its evolution.

Acknowledgements

This research was funded in part by the United States Agency for International Development, as a linkage grant to IITA and USDA-ARS, and by the United Soybean Board. We would like to thank R. Frederick for his critical review of an earlier version of this manuscript.

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